

IV. THE ISOLATION OF A SECOND STEROL FROM YEAST-FAT.

(PRELIMINARY COMMUNICATION.)

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THE isolation of a sterol from yeast was successfully accomplished by Gérard who obtained it as white crystals melting at 135–136°. Gérard [1895] regarded it as belonging to the same group as the ergosterol (m.p. 154°) prepared by Tanret [1889] from ergot. He showed that they both gave a typical colour reaction with chloroform and sulphuric acid which differentiated them from cholesterol and phytosterol and he regarded them as characteristic of the sterols occurring in the group of cryptogams. Hinsberg and Roos [1903] isolated two sterols from yeast, melting respectively at 159° and 148–149°. They regarded the lower melting sterol as a separate substance since they were unable to raise its melting point by further recrystallisation. Later Neville [1913] isolated only a substance melting at 148–149°.

Working with Thomas [1920] I investigated the various constituents of yeast-fat and obtained a crude sterol melting at 135–136°, which after being many times recrystallised melted in an open tube at 154°. Tanret [1908] found that the melting point was best taken in a sealed capillary tube filled with CO₂ as the oxidation products formed by heating ergosterol in air when the determination is made in an open tube lower the melting point. This probably accounts in part for the discrepancies as to melting point which are recorded in the literature.

We worked with a considerable number of samples of brewery yeast: from one of these only no ergosterol was isolated but in its place a white crystalline sterol melting at 97–98° and having a specific rotation of +10.1° in chloroform solution. So little material was available that we were then unable to examine it further. Since that time very many samples of yeast have been examined and in them all ergosterol has been found. I had however noticed at various times that although it is possible to obtain from yeast-fat relatively good yields of solid white sterol melting somewhere between 120° and 140°, only a comparatively small yield is obtained melting at or above 154°. It has now been found possible to separate this crude sterol, by recrystallisation from alcohol, ether and acetone into (1) the less soluble ergosterol, melting at 158.5° (in an open capillary tube), and (2) a white crystalline sterol which, after many recrystallisations, melted in an open capillary tube

at 108–109°. The two sterols appear to occur in varying proportions: in one preparation a rough estimate based on the amounts of high and low melting fractions separated after repeated recrystallisations indicated that the proportion of ergosterol was about twice that of the lower melting sterol. It will be convenient to refer to the latter as *zymosterol* from its source of origin, until more is known of its relation to ergosterol. Of these two sterols, *zymosterol* appears to be the more unstable to the action of air and light.

Optical rotation. The new *zymosterol* is dextrorotatory; the specimens originally obtained melted between 99° and 104° and gave in ether solution specific rotations for the green mercury line at 20° of +16.1°, +16.5°, and +16.8°. In chloroform solution one of these specimens gave a specific rotation of +17.3°. By many subsequent recrystallisations from ether, acetone and a mixture of alcohol and ether, a specimen was obtained melting at 108–109°, and the specific rotation of this in ether solution was +34.1°. The specimen of ergosterol melting in an open capillary tube at 158.5° gave a specific rotation $[\alpha]_{5461} = -130^\circ$ in chloroform solution. The highest value I can find recorded for the specific rotation of ergosterol is that given by Tanret for the anhydrous substance in chloroform solution, $[\alpha]_D = -132^\circ$.

It is probable that the values for both the melting point and specific rotation here given are too low, for the examination of the absorption spectrum (cp. p. 25) indicates that the *zymosterol* still contains small amounts of ergosterol, but I have not so far had sufficient material available to continue the recrystallisations until a pure specimen was obtained. From the examination of the absorption spectrum it seems that the amount of ergosterol present must be less than 5%. Since ergosterol is laevo- and *zymosterol* is dextro-rotatory, the determination of the specific rotation affords the most convenient method of following the separation of a mixture of these two substances.

Analysis. A micro-combustion of each of the two sterols was made and gave the following results for the anhydrous substances dried to constant weight in a vacuum desiccator.

5.510 mg. <i>zymosterol</i> :	5.483 mg. H ₂ O	H, 11.05 %
	17.124 „ CO ₂	C, 84.72 %
4.282 „ <i>ergosterol</i> :	4.480 „ H ₂ O	H, 11.62 %
	13.27 „ CO ₂	C, 84.54 %
Calculated for C ₂₇ H ₄₂ O		H, 10.99; C, 84.17 %

Like ergosterol, *zymosterol* is quantitatively precipitated by digitonin. It follows therefore that the estimation of the amount of ergosterol in yeast-fat, by weighing the precipitated digitonide, gives the amount of the two sterols and is not a measure of the ergosterol content.

The *acetate* of *zymosterol* was prepared by heating 0.78 g. of the sterol with 5 cc. of acetic anhydride and warming on the sand-bath for 15 minutes. After cooling and diluting the mixture, the crystals which separated were

washed with water and recrystallised from alcohol. The crystals first separating melted at 112–113°: after three recrystallisations the melting point was raised to 115°. The acetate crystallises in thin flat plates radiating from a point. The iodine value determined by Hübl's reagent was 226: the calculated value for $C_{27}H_{41}OOC.CH_3$ containing three double bonds is 179.7.

The sterol gives a modified Salkowski colour reaction: when shaken with chloroform and sulphuric acid a yellow colour is produced in the sulphuric acid layer instead of the red brown colour produced in the case of ergosterol.

The number of ethenoid linkages. It has previously been pointed out that the determination of the iodine value of ergosterol presents considerable difficulty [Smedley-MacLean and Thomas, 1921]. If Wijs solution is used to determine the iodine value, the numbers obtained are nearly twice as great as those given with Hübl's reagent. Even with the latter the values obtained for the sterols are high and vary with the excess of reagent present, the time during which the reaction mixture stands and its temperature.

From the consideration of the figures obtained for ergosterol, the presence of three ethenoid linkages in the molecule was deduced [Smedley-MacLean and Thomas, 1920], a result which has since been confirmed by Windaus from the behaviour of this sterol on hydrogenation [Windaus and Grosskopf, 1923].

In determining the iodine value of the new dextro-sterol the corresponding number for ergosterol was obtained at the same time under quite similar conditions. Thus, using Hübl's reagent, the values for a specimen of ergosterol (which melted in an open tube at 145°) and for the dextro-sterol (m.p. 96–98°) were respectively 236 and 241.2. The anhydrous sterol $C_{27}H_{42}O$ containing three ethenoid linkages requires 199.4.

Dam [1924] recommends the pyridine sulphate-bromine reagent in glacial acetic solution¹, described by Rosenmund and Kuhnhehn [1923] for the determination of the iodine value of cholesterol, and claims that when this reagent is used constant results are obtained even if considerable variations in the excess of reagent and the time of reaction take place. The method has the advantage that the reaction is ended in from 3 to 5 minutes, and Dam found that no increase in the value for cholesterol occurred even if the time was extended to 15 minutes.

0.02 g. sterol was dissolved in 5 cc. chloroform and 10 cc. of the pyridine reagent were added. After standing for 5 minutes 3.5 cc. of a 10 % solution of potassium iodide were added and the mixture was titrated with *N*/10 thio-sulphate until the brown colour had almost disappeared: 1 cc. of starch solution was then added and the titration completed. It may be noted that the chloroform solution becomes deep yellow in colour, so that the starch must be added when the *brown* colour of the iodine is still visible.

The iodine values obtained by this method were, for a sample of ergosterol

¹ The reagent is made by adding 8 g. pyridine to 10 g. concentrated H_2SO_4 in 20 cc. glacial acetic acid. A solution of 8 g. bromine in 20 cc. glacial acetic acid is added and the mixture made up with the same solvent to a litre.

(M.P. in open tube, 158.5°), 296.0 and for the zymosterol (melting at 99–101°, $[\alpha]_{5461} = +16.1^\circ$), 299.8. If the reagent was left in contact with the sterol solution for 15 minutes, considerably higher values were found and excess of the reagent seemed to produce the same effect. As far as these results go the method seems no more satisfactory than that of Hübl for the estimation of the iodine value of the highly unsaturated sterols.

From these results it is probable that the number of ethenoid linkages in ergosterol is the same as that in the zymosterol now described and further that the structure of both substances must be closely similar, since whatever be the structure other than an ethenoid linkage, which is responsible for the abnormally high iodine values obtained, it is present in both sterols alike.

Zymosterol therefore like ergosterol probably contains three ethenoid linkages.

The action of bromine. A well-cooled solution of 0.3 g. bromine in 10 cc. dry ether at 0° was slowly added to a solution of 0.25 g. zymosterol in 12.5 cc. ether. The solution was at first rapidly decolorised. The excess of bromine and ether were removed *in vacuo* and a brown oil remained giving off HBr and turning a deep green. The behaviour on bromination is very similar to that of ergosterol.

Absorption spectrum in the ultra-violet. Dr N. S. Lucas very kindly examined for me the absorption spectra in the ultra-violet region of specimens of ergosterol and zymosterol separated from the crude yeast sterol. The specimens were first examined in a dilution in alcohol of 1 in 20,000. At this dilution the characteristic absorption bands of the ergosterol spectrum were visible only in the light transmitted through the ergosterol solution. In that transmitted through the solution of zymosterol there was no evidence of any selective absorption. When the specimen of zymosterol (M.P. 108–109°) was examined in a concentration of 1 in 5000 the absorption bands in the ultra-violet region characteristic of ergosterol became visible. They were however not as marked as in a solution of ergosterol in a concentration of 1 in 100,000. It is therefore probable that in this specimen of zymosterol, ergosterol was present but to a less extent than 5 %. It seems possible that a comparison of the absorption spectra of solutions of these compounds will provide the best method of detecting the presence of ergosterol as an impurity in specimens of zymosterol.

The biological action of the zymosterol is at present being investigated.

DISCUSSION.

It is evident that two sterols exist in yeast, clearly differentiated by their difference in optical rotation.

The evidence now brought forward supports the view that zymosterol is closely related to ergosterol. Both are precipitated by digitonin; they are equally unsaturated and contain at least three ethenoid linkages. Probably the high iodine values obtained by all the methods employed are to be ascribed to some peculiarity of structure present in the sterols rather than to the

presence of additional ethenoid linkages, for even with cholesterol abnormal values are found.

In Tanret's original work on ergosterol, he isolated from ergot a second sterol melting at 144° (on the Maquenne block) and giving in ether solution a laevorotation $[\alpha]_D = -12.9^{\circ}$. He named this fungisterol and ascribed to it the formula $C_{25}H_{40}O.H_2O$; its acetate melted at 158.5° . I have so far not had an opportunity of comparing zymosterol with fungisterol. The melting points of the latter and of its acetate respectively are about 30° to 40° higher than those of the corresponding zymosterol compounds: on the other hand the specific rotation of fungisterol in ether solution is $[\alpha]_D = -12.9^{\circ}$ compared with $[\alpha]_{5461} = +34.1^{\circ}$ for zymosterol and $[\alpha]_D = -105^{\circ}$ for ergosterol. While the possibility is not excluded that fungisterol is an impure form of zymosterol, the available evidence does not support this view. A further study of fungisterol is necessary before a definite conclusion can be reached.

At present but little can be said as to the relation of the dextrorotatory zymosterol to the laevorotatory ergosterol. Windaus [1916] showed that the four isomeric dihydrocholesterols, coprosterol, β -cholestanol, pseudocholesterol and ϵ -cholestanol are all dextrorotatory. In the first two of these the steric position of the hydroxyl groups, each of which is attached to a similarly placed asymmetric carbon atom, is the same and both these sterols are precipitated by digitonin. Pseudocoprosterol and ϵ -cholestanol however are differentiated from coprosterol and β -cholestanol by the steric position of the hydroxyl group and neither of these is precipitated by digitonin. This may perhaps be regarded as an indication that in ergosterol and zymosterol the steric positions of the OH and H groups are similar.

The best known instances of dextrorotatory sterols previously described are (1) the four dihydrocholesterols referred to above, all of which are saturated, and (2) *iso*-cholesterol, about the structure of which we know very little. The existence of a dextrorotatory and highly unsaturated sterol in yeast is therefore of particular interest.

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